World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 7.409

EFFECTIVENESS TEST OF SECONDARY METABOLITES TRICHODERMA SP. AND PSEUDOMONAS FLUORESCENS AS THE BIOLOGICAL CONTROL AGENCY OF THE GANODERMA BONINENSE PATHOGENS IN THE LABORATORY

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Article Received on 14/05/2024 **Article Revised on 03/05/2024** **Article Accepted on 24/06/2024**

ABSTRACT
Ganoderma boninense is a pathogen that causes stem rot in oil palm plants. This pathogen is considered important because it can cause losses of around 50-80% per ha, can attack and spread in plant tissue from seedbeds to mature plants till plants rot, die and eventually fall. In addition, the disease has a wide host range and special structure so that it is able to survive and infect target plants. Various efforts have been made to control stem rot disease in oil palm plants but have not been completely successful. This study aims to test the effectiveness of secondary metabolites produced by Trichoderma sp. and Pseudomonas fluorescens singly or in combination as the biological control agencies pathogen G. boninense in the laboratory of the BBPPTP Medan. A completely randomized design was used to test four treatments, namely secondary metabolites of Trichoderma sp., P. fluorescens, Trichoderma sp. + P. fluorescens, and control were repeated ten times. The observation parameter was the percentage of inhibition of G. boninense colony length on PDA culture media. The research results showed that the secondary metabolites of Trichoderma sp., P. fluorescens, singly or in combination, were able to inhibit the colony length of the pathogen G. boninense which causes stem rot disease in the laboratory. The percentage of inhibition of colony length by secondary metabolites of Trichoderma sp., P. fluorescens or a combination of both respectively was 11.50%; 22.13% and 17.88%.

KEYWORDS: Stem rot, palm plants, antagonistic microbes, bioactive compound, laboratory tests.

INTRODUCTION
Oil palm (Elaeis guineensis Jacq.) is an important crop in the plantation sector because it produces the largest economic value per hectare in Indonesia (Fauzi et al., 2012). Various processed palm oil products are able to contribute to the country's foreign exchange. However, this contribution may decrease along with the decline in Indonesian palm oil production. Factors causing the decline in production include the old age of the plants, also the use of seeds that are not superior (not certified), soil fertility that is increasingly being eroded and attacks by pests (pests and diseases).

According to Yanti et al., (2019), one of the important pests that is very detrimental and attacks oil palm plants from seedlings to mature plants is stem rot. This disease is caused by the Ganoderma boninense pathogen (Semangun, 2000). Pathogens can cause losses of around 50-80% per ha, or the equivalent of 500 million USD/year. The fungus infects oil palm roots, causing tissue necrosis in both the roots and stem base (Dwipa and Nazrez, 2018). The presence of infection causes nutrient transport from the soil to be disrupted so that the 3 spear leaves do not open.

Susanto et al., (2014) said that this disease has a wide host range and special structure so that it is able to survive and infect target plants. This statement is supported by Susanto (2012) who said that no oil palm is resistant or immune to this disease, in fact infection occurs very quickly. Gardens that are fertilized with macro nutrients such as nitrogen (N), potassium (P), and potassium (K) can improve plant health. However, deficiencies in micronutrients such as boron (B), copper (Cu) and magnesium (Mg) can increase the incidence of disease (Risanda, 2008).

Control of stem rot disease is generally carried out by applying synthetic chemical fungicides. However, continuous application of fungicides can cause negative impacts not only on the environment, but also on the environment and human health (Ika, 2020). Beside that, diseases are difficult for fungicides to reach because they

are in plant tissue (Hendra, et al., 2019) causing plants to rot, die and eventually fall.

Biological control agents that are often studied are Trichoderma spp. and Pseudomonas fluorescens. Trichoderma spp. has been proven to be able to overcome various plant diseases (Munir et al., 2013; Kumar et al., 2017; Ghazanfar et al., 2018). Meanwhile, P. fluorescens is also used and is able to biologically control various plant diseases (Sivasakthi et al., 2014). Antagonistic microbes (Rashid et al., 2016) and chemicals (Muturi et al., 2017), will be directly degraded by sunlight when applied in the field, thereby limiting their capabilities. One strategy to avoid degradation of biological agents needs to be to develop the use of the secondary metabolites they produce (Mutawila et al., 2015).

Trichoderma spp. produces secondary metabolites consisting of various bioactive compounds (Mukherjee et al., 2012b; Vinale et al., 2014). P. fluorescens produces secondary metabolites that contain antibiotics and are PGPR (Plant Gowth Promoting Rhizobacter), which inhibit the growth of pathogens and stimulate plant growth (Sahu et al., 2018; Alsohim, 2020). The use of these bacteria has reportedly provided positive results on growth and production in agricultural plants (Jain & Das, 2016). However, secondary metabolites from these two microbes have never been tested either singly or in combination to inhibit the growth of pathogenic colonies of G. boninense in the laboratory. The aim of this research was to test the effectiveness of secondary metabolites produced by Trichoderma sp. and P. fluorescens either singly or in combination as the biological control agency pathogens G. boninense in the laboratory.

MATERIAL AND METHODS

Preparation of antagonist isolates

Two antagonistic microbes used, Trichoderma sp. and P. fluorescens, collection of the Balai Besar Perbenihan dan Proteksi Tanaman Perkebunan (BBPPTP) Medan. Trichoderma was grown again on PDA medium and incubated at room temperature for 5 days (Yun et al., 2017), while P. fluorescens was grown on King's B medium and incubated at room temperature for 3 days (Lamichhane & Varvaro, 2013). Specifically for Trichoderma, the antagonist is then propagated in cracked corn by washing the cracked corn thoroughly first, then putting it in 50 g of plastic, sterilizing with an autoclave at 121°C, 15 psi pressure for 30 minutes, and cooling. Trichoderma as many as 3 cork drill plugs (diameter 6 mm) were inoculated into cold sterile cracked corn in plastic using a sterile spatula, then the plastic mouth was closed with staples and incubated for 6-7 days at room temperature (Soesanto et al., 2014).

Preparation of secondary metabolites

Two plastic wraps of cracked corn (@ 50 g) that had Trichoderma growing on them were dissolved in 1 L of sterile water. Next, stir until the spores are released from the corn and filtered. The filtered water is ready for use. The medium used to produce secondary metabolites was a mixture of rice washing water and coconut water (4:1, v/v) and 10 g L^{-1} of sugar, then cooked until boiling, filtered, and put into sterile jerry cans and cooled. After the medium had cooled, 100 mL L^{-1} conidium solution was added (Soesanto et al., 2020). Next, the jerry cans were closed tightly and incubated by shaking manually until homogeneous for 14 days (Hudson et al., 2021). After incubation, the conidia density was calculated using a haemocytometer to determine the level of dilution, and a conidia density of 106 conidia mL^{-1} was obtained. The solution was then filtered using Whatman filter paper no. 1 to separate the fungal propagules from the supernatant. Next, the conidia solution was centrifuged at a speed of 9,000 rpm for 2 minutes to separate the conidia from the crude extract solution of secondary metabolites (Shehata et al., 2019). Trichoderma secondary metabolites are ready for application.

The stages of making P. fluorescens secondary metabolites are as follows; The snails are collected, then the shells are broken and the meat is taken. The meat is cleaned and weighed in 400 g L^{-1} water plus 2 g L^{-1} shrimp paste and boiled until soft (Soesanto et al., 2014). Next, the broth is filtered, placed in sterile jerry cans and cooled. Then P. fluorescens, which was harvested from King's B by adding 10 mL of sterile water, was added to the broth medium, shaker for 3 days at a speed of 150 rpm (Soesanto et al., 2014). After incubation, the spore density was calculated using multilevel dilutions, using Nutrient Agar (NA) media and obtained a density of 109 upk mL^{-1}. The supernatant was prepared by centrifuging the antagonist suspension at 5,000 rpm for 30 minutes (Decoin et al., 2014). The supernatant obtained is ready for use.

Preparation of culture media

In the laboratory testing, the culture medium used is Potato Dextrose Agar (PDA) media which is already in the form of melted flour. Take 39 grams of PDA flour, put it in 1 liter of water, cook it while stirring until it boils. Next, the PDA solution was put into 10 erlenmeyer tubes, 100 ml each and sterilized in an autoclave at 121°C, 15 psi pressure for 30 minutes.

Laboratory test

PDA solution in an erlenmeyer tube that has been sterilized in the autoclave, then put into laminar air flow. When the PDA solution begins to cool, then drop 1 mL of the secondary metabolite solution of Trichoderma sp, P. fluorescens or a combination of both into each erlenmeyer tube and stir until evenly mixed. After mixing thoroughly, 10 mL of the PDA solution was poured into a petri dish and left to harden. Then the PDA media is left for 1x24 hours to ensure it is not contaminated with other pathogens. Next, the 7 day old

pure culture of G. boninense was planted in a petri dish containing PDA media + secondary metabolite solution.

Research design

This study used a completely randomized design with secondary metabolite treatment of Trichoderma sp. (P1), P. fluorescens (P2), Trichoderma sp. + P. fluorescens (P3), and control (P0). The number of repetitions was ten; So the total was 40 petri dishes.

Observation variables

Observations were made by counting the length of colonies on PDA culture media every day, 7 observations. Measurements were carried out from the time the G. boninense pathogen was inoculated until the colony growth in the control treatment filled the petri dish. Next, the percentage of colony length inhibition was calculated using formula (1).

$$Percentage\ of\ inhibitory\ power\ (\%) = \frac{\text{colonies diameter before (cm)} - \text{colonies diameter after (cm)}}{\text{colonies diameter before (cm)}}\ x\ 100\% \ldots \quad (1)$$

Data analysis

Data were analyzed using analysis of variance at a confidence level of 95%. If the differences were significant between treatments, it was continued with DMRT at the 95% level.

RESULT AND DISCUSSION

Data on growth inhibition of G. boninense pathogen colonies on PDA media that has been treated with secondary metabolites of Trichoderma sp. and P. fluorescens was obtained from observations 7 times. Data are presented in Table 1.

Table 1: Growth inhibition (%) of G. boninense colonies (mm) pathogen on PDA media at the BBPPTP Medan laboratory (dai).

Treatment	The percentage (%) of inhibition the colony length of G. boninense on the … (dai)						
	1 Hsi	2 Hsi	3 Hsi	4 Hsi	5 Hsi	6 Hsi	7 Hsi
P0	0,00	0,00	0,00	0,00 b	0,00 c	0,00 c	0,00 c
P1	1,52	-13,81	-8,98	-2,13 b	8,38 b	8,49 b	11,50 b
P2	1,52	-4,15	1,37	9,58 a	18,32 a	21,64 a	22,13 a
P3	2,50	-15,87	-7,36	1,41 b	13,78 a	16,63 a	17,88 a

*) Number followed by the same letter in the same column are not significantly different according to BNJ at the 5% level.

Table 1, observations from 4-7 days shows that the percentage of growth inhibition of pathogenic G. boninense colonies on media treated with antagonistic microbial secondary metabolites, either alone or in combination, was significantly different when compared to the control. However, there was no significant difference between the P. fluorescens and combined treatments.

Application of secondary metabolites of Trichoderma sp. and P. fluorescens, both alone and in combination, were effective in inhibiting the growth of the pathogenic colony G. boninense, respectively, by 11.50%; 22.13% and 17.88%. This shows that the secondary metabolites of Trichoderma sp. and P. fluorescens is a biological control agency that has the ability to inhibit pathogen growth (Radder, 2019). Inhibition is carried out by dominating living places and food sources and destroying the cell walls of the antagonist fungi. Meanwhile, in untreated or control media, there was no inhibition of the growth of pathogen colonies. In fact, PDA media shows that pathogen colonies are growing day by day.

Inhibition of pathogenic colonies of G. boninense by secondary metabolites of P. fluorescens was highest because more antibiotic compounds and enzymes were produced. Secondary metabolites of P. fluorescens can produce siderophores, pterins, pyrroles, phenazines and various other antibiotic compounds which can kill and inhibit the growth of pathogens (Soesanto, 2015). Siderophores are able to bind and take up iron from the environment, thereby reducing its availability to the pathogen and preventing its growth. Pterins have the ability to suppress pathogen growth in various ways, including interfering with nucleic acid synthesis, inhibiting important enzymes, and damaging pathogen cell membranes. Pyrrole compounds have mechanisms that disrupt the function of microbial cell membranes, inhibit protein synthesis, and cause DNA damage, all of which lead to microbial death. P. fluorescens produces phenazine compounds such as phenazine-1-carbinol, phenazine-1,6-dicarbonyl, and phenazine-1,7-dicarbonyl (Sivasakthi et al. 2014). These compounds have strong antimicrobial activity against various plant pathogens, including fungi and bacteria. Phenazin works by various mechanisms, including damaging microbial cell membranes, producing free radicals that damage DNA, and inhibiting vital enzymes.

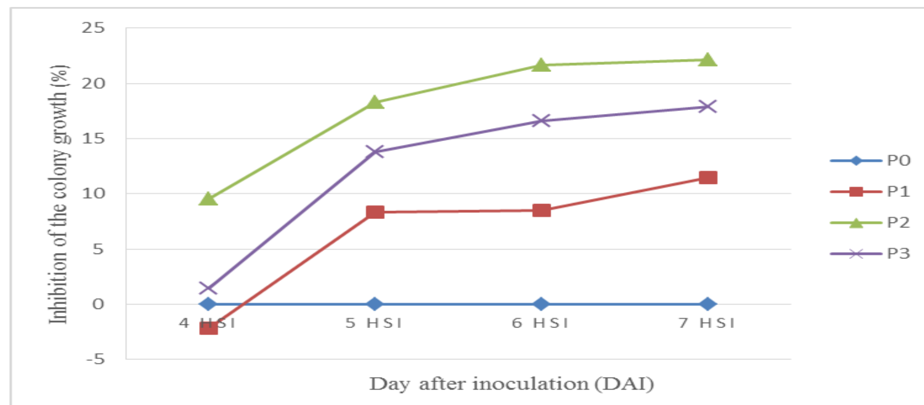

Figure 1: Percentage of inhibition of the colony growth G. boninense pathogen in each treatment.

Information: P1 = secondary metabolite of Trichoderma sp., P2 = metabolite secondary P. fluorescens, P3 = secondary metabolite of Trichoderma sp. + P. fluorescens, and P0 = control.

After secondary metabolites of P. fluorescens, Figure 1 shows the highest percentage of inhibition of colony growth G. boninense pathogen on media treated with a combination of secondary metabolites of Trichoderma and P. fluorescens. Combined secondary metabolite treatment of Trichoderma sp. and P. fluorescens showed significantly different with secondary metabolites from Trichoderma sp. although all of them experienced an increase (Table 2). This condition is thought to increase the number or quantity of bioactive compounds in the combined secondary metabolites due to synergism or the mutual neutralizing effect of the bioactive compounds in the combined secondary metabolites. In accordance with the opinion of (Ghazanfar et al., 2018) that there are interactions between bioactive compounds because the combination of several of these compounds is synergistic, additive or antagonistic.

Soesanto (2008) said that these two metabolites contain ingredients that can suppress the growth of pathogens, where the ingredients have several mechanisms. Like Trichoderma antagonistic fungi against pathogens, namely parasitism, lysis, antibiosis and space competition. This parasitic property makes the Trichoderma fungus able to effectively suppress the growth of G. boninense fungal colonies on PDA media.

Furthermore, the inhibition of G. boninense colony growth by Trichoderma secondary metabolites due to the resulting content such as antibiotic compounds such as alametichin, paracelsin, trihotoxin, gliotoxin, glioviridin, trichodermol and 1,3-β-glucanase (Arya and Perello, 2010) can destroy antagonist fungus cells. through damage to cell membrane permeability. This statement was confirmed by Vey et al., (2001), who stated that the mechanism of action of the antibiotic compounds gliotoxin and glioviridine affects and inhibits protein synthesis and disrupts the integrity of cell membranes.

Apart from antibiotics, Trichoderma secondary metabolites also contain several enzymes such as β-(1,6)-glucanase, chitinase, protease and laminarinase which can cause cell wall lysis of antagonistic fungi (Mutawilla et al., 2015). Mycoparasite Trichoderma sp. as an antagonist and kills pathogenic fungi with the combined action of bioactive compounds and hydrolysis enzymes that lyse the cell walls of pathogenic fungi (Ghazanfar et al., 2018).

Several other enzymes produced by secondary metabolites of Trichoderma sp. namely cellulolysis, xylanolysis, chitinolysis, β-1,3-glucanolysis enzymes, non-ribosomal peptides such as peptaibiotics, siderophores, gliotoxin and gliovirin as well as several lysis enzymes. The ability to produce large amounts of secondary metabolites makes Trichoderma sp. successful as a biocontrol agent.

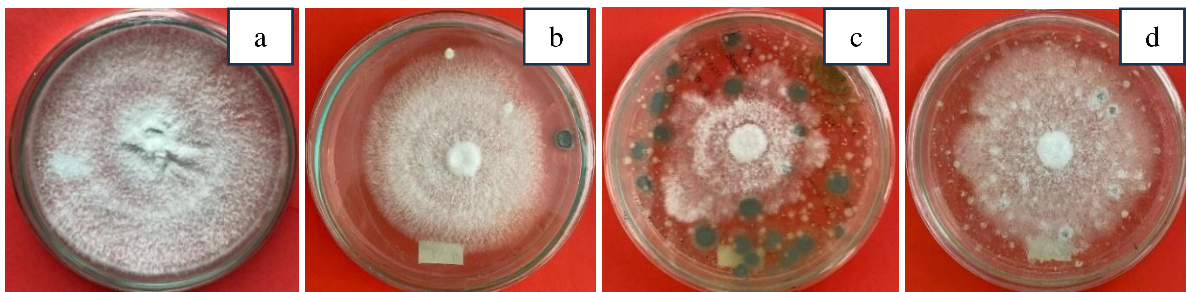

Figure 2: Growth of pathogenic colonies of G. boninense on PDA media without (a) and treated with secondary metabolites of Trichoderma sp. (b) P. fluorescens (c) and a combination of both (d) in observation VII.

Figure 2 (b, c, d) shows significant inhibition of growth of G. boninense colonies on treated PDA. Meanwhile, control media (a) continues to increase. This is because Trichoderma secondary metabolites can be used directly to control pathogens. Soesanto et al, (2020) added that Trichoderma filtrate is able to control plant pathogens on PDA media. Production of cell wall degrading enzymes, and parasitization of pathogenic cell wall contents (Mukherjee et al., 2012). Degradation of pathogen cell walls during mycoparasitism is mediated by a suite of hydrolytic enzymes including proteases, chitinase, and β-(1,6)-glucanase.

Alsohim, A.S. (2020) stated in their research that P. fluorescens also produces various other antibiotics such as phytohormones, lipopeptides, and complex compounds such as pyoluteorin and 2,4-diacetylphloroglucinol (DAPG). These antibiotics work by interfering with various vital functions in pathogenic microbial cells, such as cell membrane synthesis, protein synthesis, and nucleic acid synthesis, which ultimately causes the death of the microbe. Both secondary metabolites of Trichoderma and P. fluorescens contain bioactive compounds that function as antifungals, PGPR and induce plant resistance to attack by disease-causing pathogens (Ghazanfar et al., 2018; Sivasakthi et al., 2014).

CONCLUSION

Secondary metabolites produced by Trichoderma sp. and P. fluorescens have a significant effect in inhibiting the growth of pathogenic colonies of G. boninense in the laboratory. The percentage of the inhibition growth G. boninense pathogen colony by secondary metabolites of Trichoderma sp, P. fluorescens individually or in combination was 11.50%; 22.13% and 17.88%.

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